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# LEVAMISOLE MODULATES EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE) IN DA RATS

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*We investigated the influence of an antiparasitic drug, levamisole (2,3,5,6 - tetrahydro - 6- phenyl-imidazo (2,1 - b) thiazole - hydrochloride) with potent immunomodulatory properties on the course and development of experimental autoimmune encephalomyelitis (EAE). EAE was induced in female Dark Agouti (DA) rats aged two months by immunization with guinea pig spinal cord in complete Freunds adjuvant. Following immunization animals were subcutaneously treated every other day with 2.2 mg/kg levamisole. The course, development and characteristics of this autoimmune process were monitored as indirect indicators of immune system activity.*

*Our results indicate that in EAE levamisole exerts immunosuppressive effects when administered every other day from the moment of immunization until the end of the disease. This application regime and dose postponed the onset of the first clinical signs, shortened the duration of the disease, abrogated the severity of clinical symptoms and accelerated the recovery of sick animals. In the period of induction and during EAE, levamisole also decreased the severity of changes in the cerebral perivascular spaces. In the peripheral blood of levamisole treated animals with induced EAE, a significant increase of CD4-CD8+ T cells was demonstrated. Furthermore, all rats with induced EAE had decreased numbers of CD4+CD8- T cells in their blood. These changes were in correlation with clinical signs of EAE.*

*Key words: Dark Agouti rats, EAE, immunomodulation, levamisole*

## INTRODUCTION

For these studies we utilized DA rats, a strain which has previously been shown to be susceptible to clinical EAE following MBP-CFA immunization (Gasser *et al.* 1973, Stepaniak *et al.* 1995). As an autoimmune disease of the central nervous system induced by immunization with antigens of the nervous tissue,

EAE has been widely explored as a model for multiple sclerosis (MS) in humans. It was postulated that MS could be an autoimmune disorder but exact mechanisms of disease onset and tissue damage are still not well understood (Calder *et al.* 1989). However, one of the main characteristics of MS is a demyelinisation process which is not present in acute EAE. This form of EAE is characterized by spontaneous recovery of sick animals due to the exhaustion of self reactive T - cell clones. On the contrary, another form of the disease - chronic EAE, is characterized by massive demyelinization and can be induced by cyclosporine A treatment or immunization with nonstandard encephalitogens (i.e. myelin oligodendrocyte glycoprotein - MOG) (McCombe *et al.* 1994). This form of EAE probably resemble MS more closely (Sercarz *et al.* 1993) and because both MS and EAE involve basic immunological mechanisms the latter has been widely explored.

Levamisole is well known as a potent anthelmintic drug effective against *Haemonchus spp., Trichostrongylus spp., Ostertagia spp., Cooperia spp., Nematodirus spp, Bunostomum spp., Oesophagostomum spp and Chabertia spp.* It is recommended especially for ruminants but has some side effects in other species (Brander *et al.* 1994, Adams, 1995, Yolande 1996). Surprisingly, this drug was discovered to exert numerous immunomodulatory effects as well. There is voluminous literature concerning the immunomodulatory effects of levamisole and it was concluded that its main effect is immunostimulation in patients with suppressed immune system activity (Amery and Brynseels, 1992). Renoux and Renoux (1972) reported the first experimental data concerning levamisole action on components of the immune system. This topic is reviewed in detail elsewhere (Symoens and Rosenthal 1976, Amery and Brynseels, 1992, Goodman *et al.* 1995). The nature of levamisole action upon cells and molecules of the immune system has been intensively investigated. It was documented that levamisole induces synthesis of substances similar to thymic hormones (Renoux *et al.* 1980, Hadden 1987, Amery and Brynseels, 1992). One possible way of levamisole action could be regulation of the intracellular cGMP/cAMP ratio (Anderson *et al.* 1976, Hadden, 1977). According to the third theory, levamisole downregulates the synthesis of natural immunosuppressive factors, thus enhancing the immune response Schnaper *et al.*, Lau *et al.* 1990). One of the target cells for levamisole is the macrophage and it was reported that macrophage IL-1 secretion is augmented in the presence of this drug (Kimball, 1993).

There is not much evidence about the influence of levamisole on EAE. The first data originate from Spreafico *et al.* (1975) who demonstrated that in Lewis rats, levamisole (3 mg/kg) applied in the first four days postimmunization increased the incidence of the disease and severity of the symptoms. In another study, Ryskova *et al.* (1988) showed that levamisole (20 mg/kg) and etimisole (15 mg/kg) if applied every day after immunization abrogate clinical signs and histopathological brain lesions in guinea pigs with induced EAE. In the same study it was also documented that etimisole decreased anti MBP-antibody (Myelin Basic Protein) production while levamisole did not.

It is well known that in some clinical cases the ratio of CD4+CD8-/ CD4-CD8+ cells can be one of the indicators of immune system activity. An elevated number of CD4+CD8- cells indicates enhanced activity while elevation of CD4-CD8+ cells is a sign of decreased immune system activity (Tizzard, 1996). It is also well documented that EAE develops as a consequence of CD4+CD8- cell sensibilization with MBP (Holda and Swanberg, 1982, Burns *et al.*, 1984,

Saoudi *et al.* 1996) and that Th1 cells are probably of major significance. The role of Th2 cells is not yet clearly established (Katz *et al.*, 1995). Hauser *et al.* (1984) proved that one of the consequences of EAE in mice was decreased CD4+CD8- cell number in the peripheral blood of immunized animals. Fallis *et al.* (1987) reported that in the venous blood of Lewis rats, the number of both investigated T cell subpopulations (CD4+CD8- and CD4-CD8+) decreased even before the onset of the first clinical signs of EAE. During the recovery period the number of T cells increased. It was also postulated (Sedgwick, 1988) that CD4-CD8+ T cells are not of greater importance for EAE induction because in CD4-CD8+ depleted rats reinduction of EAE is not possible which is the same as in animals with functional CD4-CD8+ lymphocytes. Furthermore, it is not clear if during the recovery phase of EAE, the number of CD4-CD8+ cells in brain increases or their activity is suppressed. Zeine and Owens (1993) demonstrated that during the recovery period CD4-CD8+ T cells withdraw from the CNS. Vukmanovic *et al.* (1990) reported that in DA rats with induced EAE the number of CD4-CD8+ was slightly decreased in peripheral blood but significantly increased during the recovery period.

The aim of this study was to investigate the influence of levamisole treatment on the course, duration, clinical signs and severity of EAE symptoms in female DA rats. We also investigated histopathological changes in the brain of experimental animals and the distribution of CD4-CD8+ and CD4+CD8- T cells in the peripheral blood. The influence of levamisole on the immune system is of particular interest for veterinary medicine because of its widespread use as an anthelmintic drug.

## MATERIAL AND METHODS

*Animals.* Inbred Dark Agouti (DA) female rats aged 2-3 months from a breeding colony of the Medical Military Academy, Belgrade, were used for the experiments. The animals were age-matched and housed at 4-5 per cage. They were given a standard diet and tap water *ad libitum.*

*Induction of EAE.* EAE was induced in DA female rats by intradermal injection in the hind footpad of 0.1 ml of an emulsion containing guinea pig spinal cord (GPSC) homogenate (20 mg/rat) in an equal volume of complete Freunds adjuvant (CFA). CFA was made up with 8.5 parts of paraffin oil (Bayol F), 1.5 parts of an emulgant (Arlacel A) and 6 mg/ml of heat-killed *Mycobacterium tuberculosis*/rat). In addition, the rats were injected with 0.3 ml *Bordetella pertussis* vaccine ($9 \times 10^9$ microorganisms/rat) in the dorsum of the same foot (Levine and Wenk, 1965). Animals were scored daily for clinical signs of the disease on a scale from 0 to 4 defined as follows: 0 - no clinical signs, 1 - flaccid tail, 2 - weakness of hind limbs, 3 - paralysis of hind limbs and 4 - quadriplegia and moribund state (Levine and Wenk 1965). Aggregate clinical scores represent the sum of clinical signs for each day when the animal was paralyzed.

*Levamisole treatment and experimental design.* Our experiments were conducted in two separate trials. In the first trial all immunized animals (n = 40) were divided in two uniform groups. Animals from the EL group received SC injections of 2.2 mg/kg levamisole hydrochloride in 0.5 ml aqueous solution every other day starting from the day of immunization. Following immunization, the control group of animals (E) was administered the same volume of sterile saline

solution every other day. During this trial we monitored the onset of clinical signs, course of the disease and severity of symptoms. All animals were sacrificed under ether anesthesia and sections of the brain tissue were examined for the presence of perivascular infiltrates. In the second trial, 32 animals were immunized in the same way. Half of them were levamisole treated (group EL) while the other half represented a positive control group (E). Furthermore, 4 additional negative control groups consisting of 16 DA rats each were included. They consisted of animals immunized only with CFA, some of which received saline solution (group A, n=16), and some levamisole every other day (group AL, n = 16). The third and fourth group (L and C) consisted of 16 animals each, that were not immunized at all but were treated with levamisole or saline respectively. Half of these animals were sacrificed at the moment of the maximal clinical score in groups E and EL (day 14) and at the end of the recovery phase (day 21). The venous blood of these animals was examined for the presence of T cell subpopulations CD4-CD8+ and CD4+CD8- on days 14 and 21.

*Histopathological evaluation of EAE.* Animals were sacrificed 14 or 21 days postimmunization and their brains processed for staining with haematoxylin and eosin. Semithin (6 μm) serial sections of the brain were examined by light microscopy for the presence of histopathological lesions characteristic of EAE. A minimum of 12 sections per animal was examined and judged on a 0 - 4 scale as follows: 0 - no lesions, 1- small meningeal infiltrates and solitary perivascular infiltrates (lesions), 2 - meningeal infiltrates and small parenchymal lesions, 3 - multiple perivascular lesions and 4 - marked, confluent lesions with necrotic changes of brain parenchyma.

*Expression of CD4 and CD8 molecules on the lymphocytes.* Heparinized blood samples (25 UI/ml Heparin, ICN, Yugoslavia) were obtained by cardiac puncture from rats under ether anesthesia. Blood was diluted (1:1) by 0.1 M PBS containing 0.02% $NaN_3$, (pH=7.3) and layered on Ficol-Paque PLUS 1.077 gradient (Pharmacia, Biotech) prior to centrifugation (1200 rpm, 20 min.). The buffy ring was than collected and mononuclear cells washed 3 times in the same buffer (1200 rpm, 5 min, +4 °C). Following the last centrifugation the supernatant was discarded and cells resuspended in the same buffer supplemented with 2% FCS and 5% normal rat serum. The number of viable cells was estimated by trypan blue exclusion and counted in a Neubauer hemocytometer. Suspensions were then adjusted with the same PBS buffer to $1.5 \times 10^7$ cells/ml.

The number of CD4+ T cells was estimated by flow cytometry following direct unicolour labeling of CD4 molecules with mouse anti-rat CD4 - FITC monoclonal antibodies (clone W3/25, Serotec, MCA55F) of $IgG_1$ isotype (10 μl/sample in 1:50 dilution with PBS plus 0,1 $NaN_3$). For isotype control of these antibodies mouse $IgG_1$-FITC (Mouse $IgG_1$-FITC, Serotec, MCA 1209F) was used. The number of CD8 molecules was estimated by indirect unicolour immunofluorescent labeling. As primary antibodies mouse anti-rat CD8 (clone MRC OX-8, Serotec, MCA48G) IgG1 isotype (10 μl/sample, previously diluted by PBS containing 0.1% $NaN_3$ and 5% normal rat serum were used. Fluorescence labeling was performed in the second step by 10 çl of FITC - conjugated sheep anti-mouse anti- IgG serum (INEP, Zemun). This antiserum was diluted 1:20 in PBS containing 0.1% $NaN_3$ and 5% normal rat serum. In order to exclude nonspecific interactions of primary and secondary antibodies with components of the system four control samples were introduced. They were as follows: isotype

control - cells incubated with mouse $IgG_1$-FITC (Serotec, MCA 1209F), cells incubated with only primary antibodies, cells incubated with only secondary antibodies and finally cells incubated with secondary antibodies and mouse $IgG_1$-FITC (Serotec, MCA 1209F). All samples were processed on the same day by flow cytometry (FACScan; Becton Dickinson) using the Consort 30 program. Details of the FACS analyses are described elsewhere (Melamed *et al.* 1979).

*Data Analysis.* All results are expressed as mean value (X) ± standard deviation (SD). Data were analyzed by means of one-way analysis of variance (ANOVA) and Student's t-test.

## RESULTS

*Trial I:* Clinical data for EAE in the female DA rats immunized with GPSC (E) and treated with 2.2 mg/kg levamisole every other day (EL) are presented in Tables 1 and 2. The results showed that EAE was induced in all animals (incidence 100%) but the severity of the symptoms differed significantly between levamisole treated and the control group. In the levamisole treated group (EL) only two animals showed signs of quadriplegia while in the control group 9 animals exibited the most serious clinical stage (Table 1). Levamisole treatment significantly postponed the onset of the clinical signs, shortened the duration of the disease and decreased the aggregate clinical score in sick animals. Moreover, in this group of DA rats, the mean clinical score was decreased and the peak day was delayed (Table 2).

Table 1. EAE incidence and clinical signs in control (E) and levamisole treated female DA rats (EL) following immunization with GPSC in complete Freunds adjuvant

| Group | n | EAE (%) | Number of animals with clinical signs | | | | | % of para- and quadriplegia |
|---|---|---|---|---|---|---|---|---|
| | | | 0 | + | ++ | +++ | ++++ | |
| E | 20 | 100 | 0 | 1 | 1 | 9 | 9 | 90 |
| EL | 20 | 100 | 0 | 0 | 4 | 14 | 2 | 80 |

Table 2. EAE parameters in control (E) and levamisole treated female DA rats (EL) following immunization with GPSC in complete Freunds adjuvant (n=20)

| Group | Day of EAE onset | Peak day | EAE duration (days) | Maximal score | Aggregate score |
|---|---|---|---|---|---|
| E | 8.3±1.2 | 10.6±1.2 | 7.2±1.4 | 3.3±0.8 | 16.7±4.5 |
| EL | 10.1±1.4[a] | 11.3±1.5 | 4.7±1.4[a] | 2.9±0.6 | 10.2±3.98[a] |

Examination of the haematoxylin-eosin stained brain sections from the animals included in this trial revealed the inhibitory effect of levamisole on the development of brain lesions. In the levamisole treated group, brain lesions were documented only in 40% of the animals. In this group of animals lesions were less severe and these differences were statistically significant (Table 3).

Table 3. Perivascular mononuclear infiltrates in control (E) and levamisole treated female DA rats (EL) following immunization with GPSC in complete Freunds adjuvant

| Group | n | Animals with brain lesions (%) | Number of animals and severity of brain lesions | | | | | X±SD |
|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | |
| E | 10 | 100 | 1 | 1 | 4 | 3 | 1 | 90 |
| EL | 10 | 100 | 6 | 1 | 2 | 1 | 0 | 80 |

The course of the disease in the levamisole treated and control groups of DA female rats with induced EAE is presented in Figure 1. It is evident that from the day of onset, the mean clinical score was always lower in the levamisole treated group. Furthermore, the peak day (when maximal clinical symptoms were expressed), was delayed due to the influence of levamisole.

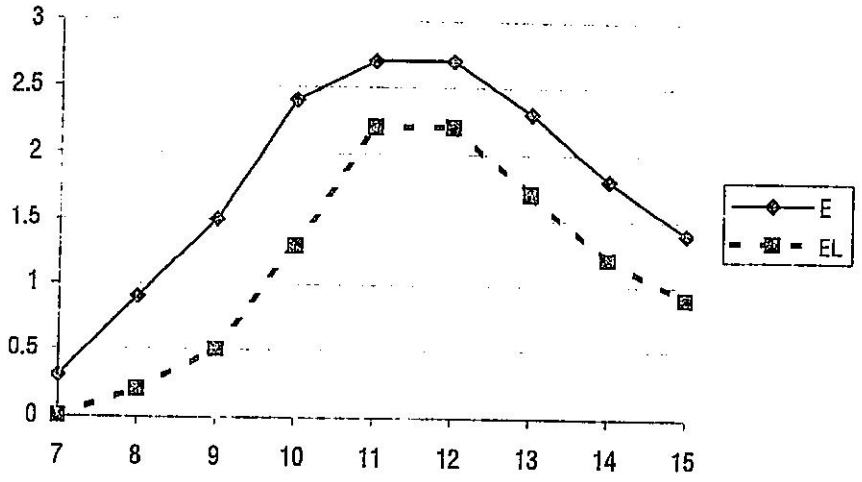


Figure 1. Mean clinical score of EAE in the levamisole treated (LE) group (n=20) and control (E) group (n=20)

Figure 2. shows typical, well developed, brain lesions in DA rats with induced EAE characterized by massive perivascular mononuclear infiltrates and signs of necrosis.

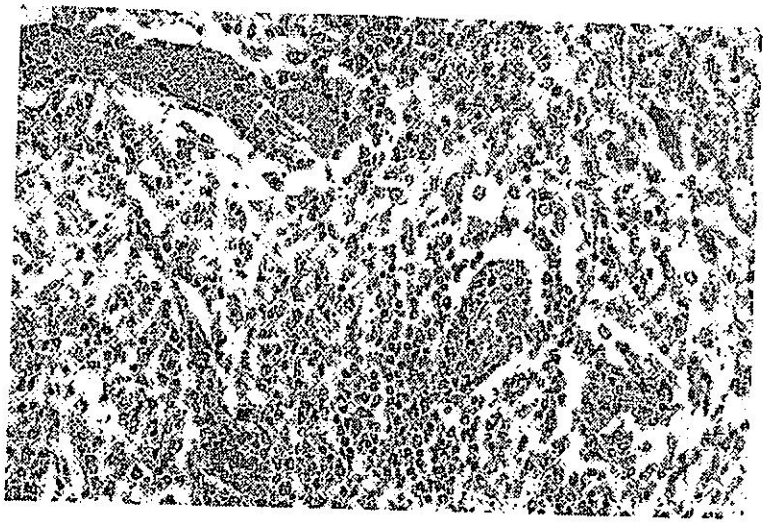

Figure 2. Massive perivascular mononuclear infiltrates and signs of necrosis in the brain sections of DA female rats with induced EAE (++++), HE, 400 X

*Trial II:* In the second trial, the same characteristics of EAE in the levamisole treated and control group were observed (data not shown here). In order to estimate the influence of all substances used in EAE induction on the number of CD4+CD8- and CD4-CD8+ cells, four additional groups were included (A, L, AL and C) as described in the Material and methods section. Half of the animals were sacrificed on day 14, when maximal clinical signs were expressed, while the other half was sacrificed one week later during the remission phase.

The number (%) of T cells in the peripheral blood of all animals obtained by FACS analyses, on day 14, is presented in Figure 3. In all negative control groups

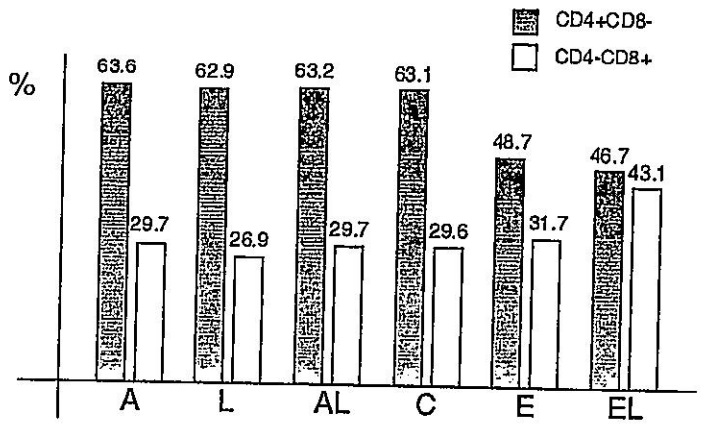


Figure 3. Mean values for relative numbers (%) of CD4+CD8- and CD4-CD8+ T cells in the peripheral blood of female DA rats on day 14 postimmunization

(animals nonimmunized with encephalitogen - A, L, AL and C) the relative number of CD4+CD8- T cells did not differ significantly. However, in groups E and EL, their level was significantly lower. The decrease of the CD4+CD8- cells was more marked in the EL group. Between the negative control groups, the number of CD4-CD8+ did not differ either but the relative number of these cells was significantly increased in groups E and EL. Again, the changes were more expressed in the levamisole treated animals (EL group).

The number (%) of T cells in the peripheral blood of all animals on day 21 is presented in Figure 4. As on day 14, in all negative control groups (A, L, AL and C) the relative number of CD4+CD8-T cells was similar but significantly decreased in groups E and EL, even more than on the day 14. The relative number of CD4-CD8+ did not differ between the negative control groups and group E either. The number of CD4-CD8+ was significantly increased only in group EL.

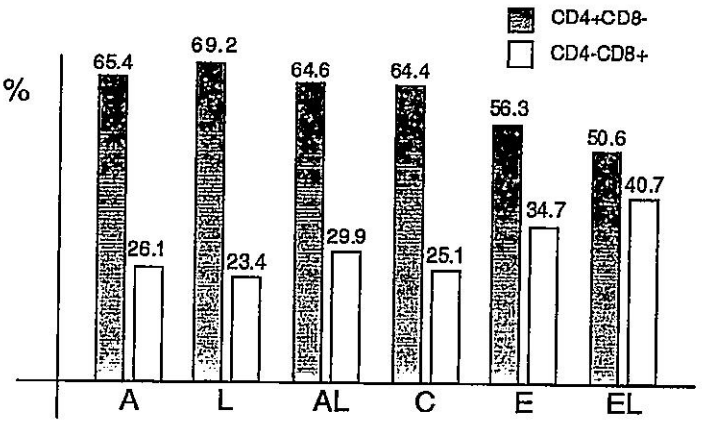


Figure 4. Mean values for relative numbers (%) of CD4+CD8- and CD4-CD8+ T cells in the peripheral blood of female DA rats on day 21 postimmunization

## DISCUSSION

Various inbred strains of rats differ in their susceptibility to encephalitogens and in the DA strain it is possible to induce EAE by a single heterologous or homologous nervous tissue injection with an adjuvant. Our results concerning EAE characteristics, correlate with the findings of Vukmanović *et al.* (1990) and Arsov *et al* (1995) who demonstrated a similar course and duration of the disease in DA rats. In our study, the levamisole treated group of rats also developed acute EAE but parameters of the disease were significantly different. Levamisole treatment postponed the onset of the clinical symptoms, shortened the duration of the disease and decreased the aggregate clinical score in sick animals. In this group of DA rats, the mean clinical score was decreased and the peak day was delayed (Table 2). These results are in agreement with findings of Ryskova *et al.* (1988) who reported suppressive effects of levamisole applied in the phase of induction on EAE development in guinea pigs. The same authors demonstrated decreased morbidity and mortality rates as well as a decreased late

hypersensitivity reaction to MBP. However, in our experiments the mortality rate was small (data not shown) because the dose of the levamisole used was much lower (2.2 vs. 20 mg/kg). In addition, another group of authors (Hierholzer and Kuwert, 1975) noted the abrogating influence of levamisole on EAE course and development. Interestingly, the first results about the influence of levamisole on EAE (Spreafico *et al. 1975) indicated potentiating effects of this drug if applied in a dose similar to ours (3 mg/kg).*

Ryskova *et al.* (1988) reported that levamisole treatment of guinea pigs with induced EAE decreased the severity of the brain lesions and similar findings were obtained in our experiments. Moreover, clinical signs of EAE in our study, correlated well with the degree of histopathological brain lesions. There is particular disagreement in the literature concerning this problem and some authors stated that histopathological changes (perivascular mononuclear cell infiltrates) in the brains of rats with induced EAE do not always correspond to the clinical signs of the disease (Krenger *et al.* 1986, Perry and Gordon, 1988).

In our second trial, it was documented that in the peripheral blood of DA female rats with induced EAE, the number of CD4+CD8- was significantly reduced in both levamisole treated and non-treated groups in comparison to the values obtained for the nonimmunized animals. Neither adjuvant nor levamisole alone nor their combination altered the number of CD4+CD8- cells. As previously stated nearly the same values were obtained on days 14 and 21. On the contrary, the number of CD4-CD8+ T cells was significantly increased in immunized animals (except for group E on day 21) and especially in the levamisole treated group. Our findings are in agreement with the results of Vukmanović *et al.* (1990) who also documented that in EAE, the balance between lymphocyte subpopulations is altered. The same authors found an increased number of CD4-CD8+ cells in the peripheral blood during the period of recovery. Our results confirm this finding and, in addition, we observed an even higher increase in the levamisole treated group. Decrease of the CD4+CD8- T cell population in the peripheral blood could be ascribed to increased migration into the target tissue (Hauser *et al.* 1984). The results of Fallis *et al.* (1987) confirm that even before the EAE manifestation in Lewis rats the number of both CD4-CD8+ and CD4+CD8- T cells was decreased in the peripheral blood. In our experiments, a decrease of CD4-CD8+ cells was not found on days 14 and 21. Zeine and Owens (1993) demonstrated that during the period of recovery the number of CD4+CD8- cells decreased in nervous tissue. There is clinical evidence that levamisole applied to patients with multiple sclerosis can normalize the CD4+/CD8+ index (Sun *et al.* 1994).

The immunosuppressive effect of levamisole in acute EAE was a consistent finding in our experiments and there are several mechanisms that might be operative. Sampson and Lui (1976) stated that, apart from its stimulatory role, levamisole can enhance activity of T c/s cells. Another possibility is conected to the role of histamine in EAE, because increased release of this mediator in the CNS potentates EAE symptoms (Stanley *et al.* 1990), while levamisole can neutralize histamine effects (De Cock *et al.* 1978). It was also documented that pentoxifilline can exert protective effects in an EAE model (Nataf *et al.* 1993) by changing the cAMP/cGMP balance in cells, thus blocking TNF - synthesis. This effect was also ascribed to levamisole (Hadden, 1977). Recently, it was emphasized that levamisole contributes to the regulation of the immune system by stimulating depressed functions and also by suppressing enhanced activity.

The effects of levamisole are often compared to thymic peptides because of its ability to correct an impaired CD4+/CD8+ index (Sibirjak *et al.* 1999).

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## MODULACIJA EKSPERIMENTALNOG AUTOIMUNOG ENCEFALOMIJELITISA (EAE) DA PACOVA LEVAMIZOLOM

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SADRŽAJ

U ovom radu su prikazani rezultati ispitivanja anthelmintika levamizola (2,3,5,6 tetrahidro - 6 - fenil - imidazo (2,1 - b) tiazol hidrohlorida) sa sna'nim imunomodulatornim svojstvima na tok i razvoj eksperimentalnog autoimunog encefalomijelitisa (EAE). EAE je indukovan imunizacijom ženki pacova soja DA (Dark Agouti) starih dva meseca pomoću homogenata kičmene mo'dine zamorčeta u kompletnom Freundovom adjuvansu. Posle imunizacije, životinje su

tretirane subkutanim injekcijama levamizola (2.2 mg/kg) svaki drugi dan a praćeni su tok, razvoj i karakteristike ovog autoimunog oboljenja kao indirektni indikatori aktivnosti imunološkog sistema. Postignuti rezultati ukazuju da levamizol ispoljava imunosupresivno delovanje u modelu EAE ako se aplikuje svaki drugi dan od momenta imunizacije do kraja bolesti. Primenjena doza i režim aplikacije odlo'ili su momenat pojavljivanja prvih kliničkih simptoma, skratili trajanje bolesti, ublažili ispoljavanje simptoma i ubrzali oporavak bolesnih životinja. U periodu indukcije i tokom EAE-a levamizol je smanjio stepen promena u cerebralnim perivaskularnim prostorima. U venskoj krvi ženki pacova sa indukovanim EAE i tretiranim levamizolom uočeno je značajno povećanje broja CD4-CD8+ T ćelija. Osim toga, u obe imunizovane grupe životinja zapaženo je smanjenje broja CD4+CD8- ćelija. Ove promene su bile u skladu sa kliničkom slikom bolesti.